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Research Article

Developmental Anomalies and Oxidative Stress Responses in Zebrafish (*Danio Rerio*) Following Embryonic Exposure to Human Pharmaceuticals

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The discharge of human pharmaceuticals via wastewater treatment plants represents a major threat to non-target aquatic organisms since they are continually exposed throughout their lifespan. The individual effects of the anaesthetic, lidocaine; the cytostatics, ifosfamide and cyclophosphamide; and the antimicrobials, sulfamethoxazole, amoxicillin and erythromycin on 24 hpf (hours post fertilization) zebrafish (*Danio rerio*) following a 96-h exposure was investigated by evaluating embryonic development, catalase (CAT) enzyme activity, and the gene expressions of CAT, cytosolic superoxide dismutase (SOD1), and mitochondrial superoxide dismutase (SOD2). Lidocaine, cyclophosphamide and sulfamethoxazole induced neurotoxicity (scoliosis, tail malformation) and cardiotoxicity (pericardial edema, bradycardia) in the zebrafish which correlate with their adverse effects in mammals. These observations were linked to oxidative stress as indicated by the significant alteration of CAT activity and amounts of transcripts of SOD1, SOD2, and CAT. The CAT activity and gene expressions of the antioxidants were significantly upregulated at sublethal and levels of ifosfamide, lidocaine, cyclophosphamide, and erythromycin non-toxic to embryonic development in this study which suggest that these antioxidants may play a role in the prevention of teratogenicity in the larvae. The multi-level effect approach adopted in this study provides a better understanding of the mechanisms of toxicity employed by lidocaine, cyclophosphamide and sulfamethoxazole towards zebrafish. This study shows the importance of evaluating stress biomarkers and emphasizes the need for chronic studies in assessing pharmaceutical effects on aquatic organisms.

Keywords: Zebrafish; Pharmaceuticals; Developmental toxicity; Antioxidants; Biomarkers; Gene expression.

INTRODUCTION

The discharge of human pharmaceuticals via wastewater is an ongoing concern since they represent a long-term threat to the non-target aquatic organisms. Although, industrial and municipal wastewaters undergo purification in wastewater treatment plants (WWTP), complete removal of many pharmaceuticals is not ensured (Kostich *et al*., 2014; Fernandez-Lopez *et al*., 2016) so significant concentrations of some pharmaceuticals can occur in effluents, groundwater, surface waters and also in drinking water (Yang *et al*., 2017; Comber *et al*., 2018).

The potential impact of any toxicant in ecosystems requires the observation of effects at different levels of biological organization (Lemos *et al*., 2010; Ferreira *et al*., 2015). Many of the studies on the ecotoxic effects of pharmaceuticals are focused on the organismal or higher levels. However, at such levels alone, the mechanisms of toxicity of the drugs are poorly understood and the predictive ability of measurements done at these levels is limited (Verslycke *et al*., 2004). Over the last decades, biomarkers at suborganismal levels have been considered viable measures of responses to stressors (Huggett *et al*., 1992). Several biomarkers including those indicative of oxidative stress have been used at the molecular and cellular levels as efficient tools due to their sensitivity,

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quickness, and accurate relationship between toxicant exposure and respective biological response (Connon *et al*., 2012; Mao *et al*., 2020).

Xenobiotics have been shown to exert various adverse effects on the health of zebrafish (Albalawi *et al*., 2018; Sachett *et al*., 2018; Patibandla *et al*., 2018; Vigano *et al*., 2020). Effects of ibuprofen, ketoprofen, diclofenac, paracetamol, fluoxetine and atenolol on zebrafish development and several biomarkers indicative of adverse effects have been investigated (Diniz *et al*., 2015; Xia *et al*., 2017; Nowakowska *et al*., 2020). Zebrafish embryos are an attractive model for studies of developmental toxicity of chemicals both for human and environmental risk assessment (Mesquita *et al*., 2017; Cassar *et al*., 2019). The rapid development, large number of offspring, optical transparency of the embryos and availability of a full genome sequence are some of the advantageous characteristics of this model (Carlsson *et al*., 2013). In addition, pharmacologic responses of zebrafish to well-characterized cardiotoxins are similar to responses in humans, making it an excellent model for cardiotoxicity assessment (Zhu *et al*., 2014).

Due to the higher pharmaceutical concentrations in hospital effluents than other effluents, a separate treatment of hospital wastewater prior to discharge into the sewage system has been highly recommended (Verlicchi *et al*., 2012; Helwig *et al*., 2013). This study examined six individual pharmaceuticals; lidocaine LDC, cyclophosphamide CYC, ifosfamide IFS, sulfamethoxazole SUF, amoxicillin AMX, and erythromycin ERY selected from a wide range of pharmaceuticals monitored in hospital wastewater in the European Union funded PILLS project (Helwig *et al*., 2013) from which the present study emanated. Selection was based on their hospital contribution, European wide usage and persistence in the environment (Helwig *et al*., 2013). LDC, a local anesthetic used in dental surgery, inhibits sodium influxes required for the initiation and conduction of nerve impulses (Short *et al*., 1988). CYC and IFS are anticancer agents and act by preventing cell division by cross-linking DNA strands and inhibiting DNA synthesis (Zounkova *et al*., 2007). The antibacterials, SUF, ERY and AMX inhibit the synthesis of folic acid, protein, and cell wall in bacteria respectively (van der Grinten *et al*., 2010; Gonzalez-Pleiter *et al*., 2013; Magdaleno *et al*., 2015).

This study was aimed at linking the selected pharmaceutical effects in zebrafish at the molecular and cellular levels to an outcome at the organismal level of organization, for example embryonic development, elucidating the mechanisms of toxicity of these compounds. This study was therefore carried out to investigate the lethal and sublethal effects of LDC, CYC, IFS, SUF, AMX and ERY on the embryonic development of 24 hpf zebrafish (*Danio rerio*); and the activity and gene expression levels of antioxidative enzymes; catalase, CAT and superoxide dismutases, SOD1 and SOD2.

MATERIALS AND METHODS

Test chemicals

Stock solutions of LDC (CAS no. 137-58-6), IFS (CAS no. 3778-73-2), CYC monohydrate (CAS no. 6055-19-2), SUF (CAS no. 723-46-6), ERY hydrate (CAS no. 114-07-8), and AMX (CAS no. 26787-78-0) purchased from Sigma with ≥ 95% purity were prepared directly by diluting in E3 embryo medium (NaCl, 292 mg/L; KCl, 13 mg/L; $CaCl_2$, 44 mg/L; $MgSO_4$, 81 mg/L; and $NaHCO_3$, 192 mg/L in deionized water plus 200 µL of 0.05% methylene blue for fungicidal effects). Stocks were kept refrigerated and protected from light.

Zebrafish husbandry and egg collection

AB strain adult zebrafish (*D. rerio*) reared in Glasgow Caledonian University Fish Facility were maintained in a flow-through system of carbon filtered tap-water (pH = 7.5; Total hardness = 150 mg/L $CaCO_3$; conductivity = 420 µS/cm). Temperature was kept at 28^{o}C and the photoperiod was 14:10 h light: darkness. Fish were fed twice daily with commercial flakes as staple food supplemented with brine shrimps (OECD, 2006). Adult zebrafish were placed in the breeding tanks on the afternoon of the day before egg collection with each tank containing two females and one male. Eggs were collected from the different tanks within 30-60 min after natural mating, pooled and rinsed thoroughly and eggs were checked under a microscope (AR-zoom, Novex, Holland); unfertilized or injured and dead eggs were removed and discarded. Fertilized eggs were kept in an incubator at 28^{o}C until 24 hpf.

Embryo development assay

Tests were carried out in accordance with the fish embryo toxicity (FET) test in the OECD draft guideline (Test no. 236; OECD, 2006). Following range finding experiments, where lethal concentrations were determined, fertilized embryos were exposed to varying concentrations of the pharmaceuticals (diluted in E3 medium): 0.11 to 0.89 mM LDC; 0.31 to 2.3 mM IFS; 0.09 to 1.58 mM SUF; 0.017 to 0.272 mM ERY; 0.41 to 2.73 mM AMX; and 0.76 to 4.59 mM CYC. The pharmaceutical ranges were selected to include sublethal concentrations with the aim of collecting materials for biomarker analysis.

Ten 24 hpf embryos per concentration were exposed to the test pharmaceuticals and the control (containing E3 medium only) in 24-well plates (BioLite, Thermo Fisher Scientific, UK) containing a final volume of 2 ml per well. Tests were performed in triplicates and exposures were carried out in an incubator at 28°C and run for 96 h without medium change. The plates were sealed with self-adhesive foil (MicroAmp® optical adhesive film, Applied Biosystems, UK) to prevent evaporation. Assays were not done concurrently except for SUF and ERY.

Embryos were observed daily under a cell imaging system in bright field configuration (EVOS® Fluorescence Cell Imaging System, Life Technologies Corporation, CA, USA) using a magnification of 4 x for embryos and 2 x for hatched juvenile zebrafish. Mortality and abnormalities including spine deformation or scoliosis, malformation of the tail and tail tip, yolk sac edema, pericardial edema, side-laying of larvae were noted at different hpf (Table 1). Embryonic heartbeat in zebrafish is first detectable at 24 hpf and is an important indicator of health (AshaRani *et al*., 2008) and thus the embryos and juveniles were considered dead when no heartbeat was observed. In addition, embryo heart rate was monitored from 48 hpf using direct microscopic observation at 4 x or 10 x magnification over 60 s for a minimum of 15 embryos at each concentration. Hatching success was assessed at 48 hpf, which is the average time at which zebrafish embryos hatch (Westerfield, 2000). The pH of control and the highest concentration of each pharmaceutical were measured at the commencement and end of the experiments and found to be within 0.6 units. Chemical analysis of exposure concentrations kept in an identical setup in the absence of embryos was also performed. Samples were taken at 0, 24, 72 and 96 h and stored at -20°C until analysis.

At 120 hpf, five to eight embryos per replicate were pooled together in an eppendorf tube and euthanized by the addition of the anaesthetic, 0.1 % MS-222 (ethyl 3-aminobenzoate methanesulfonate, tricaine, Sigma Aldrich) solution (Timme-Laragy *et al*., 2009; Bartoskova *et al*., 2014). For enzymatic and molecular analysis, the euthanized zebrafish larvae were flash frozen in liquid nitrogen and kept at -80°C.

Table 1. Endpoints observable in zebrafish embryos at different hours post fertilization

Endpoint	Description	48 hpf	72 hpf	120 hpf
Lethal categorical				
Coagulation	Embryo is coagulated with no structures	+	+	+
Lack of heart beats	Embryo has no visible heartbeat	+	+	+
Sublethal categorical				
Tail malformation	Tail is shorter than normal or curved	+	+	+
Tail tip malformation	Tail tip is curved	+	+	+
Yolk sac edema	Abnormal swelling of the yolk sac	+	+	+
Pericardial edema	Abnormal swelling of the pericardial cavity	+	+	+
Bent spine/scoliosis	Curvature or twisting of the spinal cord	+	+	+
Partial dechorionation	Larva head is trapped in chorion	+	+	+
Side-laying	Larvae lie on side (not actively swimming)			+
Unhatched	Embryo alive but unhatched at the time observed	+	+	+
Sublethal continuous				
Heart rate	Number of heart beats are counted during 1 min	+	+	+

hpf: hours post fertilization

Enzyme extraction and protein quantitation

The samples frozen for enzymological study were allowed to thaw on ice and then homogenised using a bead beater (FastPrep®-24, MP Biomedicals, France) in tubes containing 0.3 mL sodium phosphate buffer (50 mM, pH 7.5) and 1 mm glass beads (BioSpec products, UK) for 60 s at 6.5 ms^{-1}. Proteins were extracted in 0.7 ml of the sodium phosphate buffer, and tubes centrifuged at 12,000 rpm for 15 min at 4°C to separate the post mitochondrial supernatant (Oliveira *et al*., 2013) which was then removed, transferred to new tubes and kept at -80°C for a later determination of CAT activity. Total protein content in the mitochondrial fraction was determined using Bradford's reagent (Bradford, 1976). The absorbance was measured at 595 nm using bovine serum albumin as standard.

CAT activity analysis

Catalase activity was evaluated spectrophotometrically (UVmini-1240, SHIMADZU) by measuring the consumption or decrease in absorbance of H_2O_2 every 15 s at 240 nm for 2 min using an extinction coefficient of 40 $mM^{-1}cm^{-1}$ for H_2O_2 (Aebi, 1984). The test medium contained 750 µL of sodium phosphate buffer (50 mM, pH 7.5), 100 µL of H_2O_2 (200 mM) (30% w/w, PERDROGEN™, Sigma Aldrich) and 150 µL of enzymatic extract in a final volume of 1 mL. The blank contained 900 µL sodium phosphate buffer and 100 µL H_2O_2.

Total RNA extraction & reverse transcription

Total RNA was extracted using TRI Reagent® (Sigma, UK) according to the manufacturer's protocol. RNA quantity and quality were analyzed spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific, DE, USA). The High-Capacity cDNA synthesis kit with RNase inhibitor for Reverse Transcription (Applied Biosystems) was used according to the manufacturer's instructions using 1 µg of the RNA, random primers, dNTP, reverse transcriptase, and RNase inhibitor and carried out in a thermal cycler (Applied Biosystems® 2720, UK) at 37°C for 120 min. Resulting cDNA was diluted to a working concentration 20 ng/µL.

Quantitative real-time PCR

The β-actin (reference or housekeeping gene), SOD1, SOD2 and CAT primers, published previously (Timme-Laragy *et al*., 2009; Wan and Chan, 2010), were purchased from Integrated DNA Technology (IDT), UK. Primer efficiencies were tested to ensure the housekeeping and target genes amplified at the same rate. The nucleotide sequences employed are listed in Table 2. A 30 µL reaction contained 15 µL of 2X Platinum SYBR Green qPCR SuperMix-UDG with ROX kit (Invitrogen, Life Technologies, UK), 0.6 µL of each 10 µM primer, 6 µL of diluted cDNA (20 ng/µL reaction) and 7.8 µL nuclease-free H_2O. Quantitative real-time PCR was carried out in a Bio-Rad CFX96 Real-Time PCR Detection System (C1000™ Bio-Rad, UK) under the following thermal cycle: 2 min at 50°C (UDG incubation), followed by 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, then 15 s at 60°C followed by a melting curve. All samples were run in triplicates. qRT-PCR data were analyzed using the Bio-Rad Sequence Detection System (Bio-Rad).

The comparative C_T method, $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) was used to determine average fold induction of messenger RNA (mRNA) by comparing the C_T of the target gene to that of the reference gene. Reference gene expression was not altered by treatment. The amplified DNA or amplicon was evaluated by gel electrophoresis using MIDORI^{GREEN} DNA stain (Anachem, UK). A GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, UK) was run alongside PCR products in 1X Tris-acetate-EDTA buffer on agarose gel (2%) at 90 volts for 50 min using a Bio-Rad Gel electrophoresis system.

Table 2. Primers used and GenBank accession numbers for qRT-PCR in zebrafish

Gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	AF057040	ACATCCGTAAGGACCTG	GGTCGTTCGTTTGAATCTC
SOD1	Y12236	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG
SOD2	AW07696	CTAGCCCGCTGACATTACATC	TTGCCCACATAGAAATGCAC
CAT	AF170069	TGAGGCTGGGTCATCAGATA	AAAGACGGAAACAGAAGCGT

Pharmaceutical analysis

Samples were filtered through 0.2 µm cellulose filter prior to analysis. Liquid chromatography mass spectrometry (LC-MS/MS) was used to determine the actual concentrations of the pharmaceuticals. The mass spectrometer used for the analysis was a Thermo Scientific Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, England) equipped with an Atlantis dC18 chromatography column operating in positive ion mode. For each sample, 10 µl was injected using an auto-sampler. The mobile phase was 10 mmol ammonium formate (adjusted to pH = 3.5 by formic acid), and acetonitrile. A gradient elution technique was used and the flow rate was 0.3 mL min^{-1}.

All detections were performed by mass spectrometry, in which the m/*z* transition for the drugs were as follows: 261→153.9 for IFS, 261→139.9 for CYC, 254→155.9 for SUF, 734.47→158.1 for ERY, 366→349 for AMX, and 235→86.3 for LDC and concentrations determined using a 12-point calibration curve prepared using standard solutions.

Validity criteria and statistical analysis

Egg batches were only used if fertilization rates were ≥ 70%. An assay was considered valid if the controls showed < 10% sublethal categorical and/or lethal effects at 120 hpf (OECD, 2006). The lethal concentrations were calculated using probit analysis. For other responses, independent sample t test and/or analysis of variance (one-way ANOVA) was performed after testing for normality and homogeneity of variance in treatment groups using Shapiro Wilk ($p < 0.05$) and Levene's test ($p < 0.05$) respectively. Post-hoc, Dunnett test or Games-Howell test (SPSS® v22) was used to assess the significant differences among the test groups with $p < 0.05$ as the level of significance.

RESULTS

The 24 hpf embryos that were exposed to E3 medium only (controls) underwent normal development and all the control groups fulfilled the acceptance criteria of < 10% affected larvae at 120 hpf. The measured concentrations of the pharmaceuticals in this study were within 85 to 116% of the nominal concentrations and as a result, the nominal values were taken as effect concentrations and used in the data analysis in accordance with OECD guideline.

Mortality

With all the pharmaceuticals examined no significant mortality to the zebrafish embryos was recorded below 100 mg/L. The calculation of LC_{50} and LC_{10} values was only possible for LDC and CYC with a 96 h LC_{50} of 0.71 mM and 2.16 mM and a 96 h LC_{10} of 0.62 mM and 1.51 mM respectively.

LDC was the most toxic compound in this study with the highest concentrations (0.77-0.89 mM) causing mortality ($p < 0.01$) in 50-90% of the embryos after 24 h (Fig. 1a). Whereas, mortality was not observed in the embryos exposed to the highest CYC concentrations (3.44-4.59 mM) until after 72 h with 30-80% of embryos affected (Fig. 1b). For the remaining pharmaceuticals, < 10% of the larvae ($p > 0.05$) demonstrated mortality after 96 h.

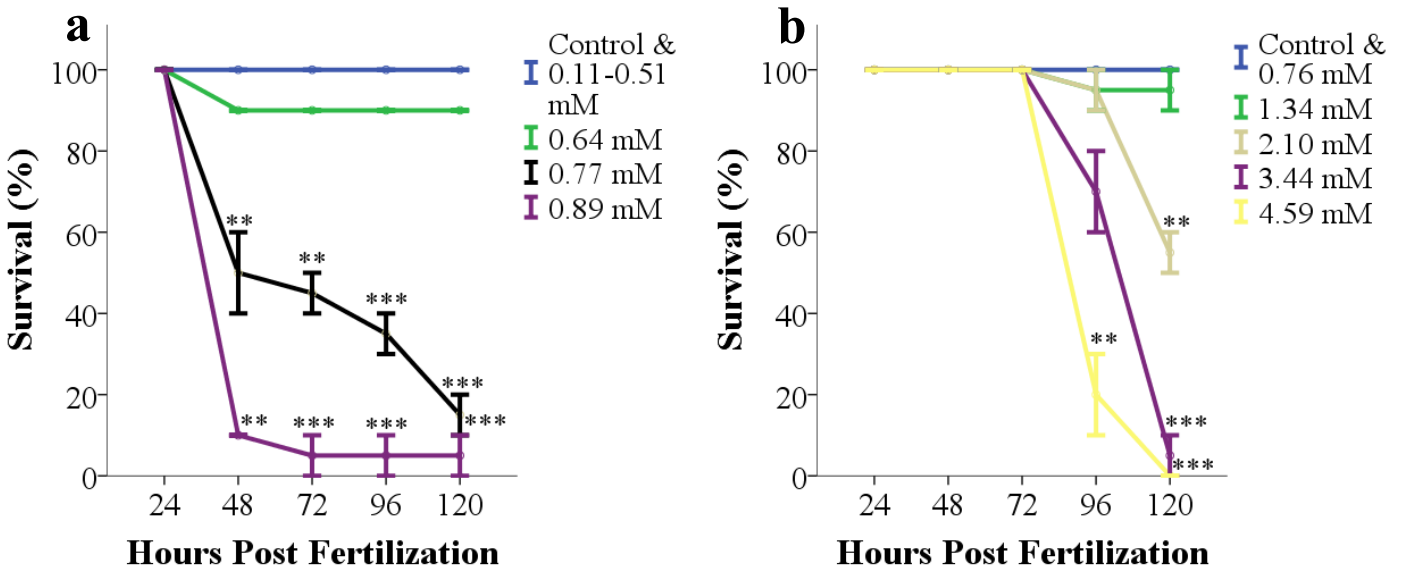

Figure 1. Survival rate of 24 hpf zebrafish embryos exposed to (a) lidocaine, and (b) cyclophosphamide for 96 h. $^{**}p < 0.01$, $^{***}p < 0.001$. Error bar represents SE.

Developmental effects

Some important developmental defects such as pericardial edema, edema of the yolk sac, bent spine or scoliosis, and malformed tail or tail tip, were induced by the pharmaceuticals. Fig. 2 shows some of the noticeable abnormalities at different stages of zebrafish development in this study.

Lidocaine

Exposure to LDC enhanced hatching of the embryos. At 48 h, only 35% of the embryos hatched in the control group but in the LDC treatments from 0.11 mM to 0.51 mM (sublethal concentrations) more than 70% of the alive embryos had hatched ($p < 0.01$) (Fig. 3a). In addition, 55.5% of the alive embryos hatched in the 0.64 mM treatment at 48 h, although this was not significantly different to the control group. Associated with the rapid hatching in the sublethal treatments (0.25-0.51 mM), significant behavioural and developmental effects were observed ($p < 0.05$) (Fig. 4a and 5a) whereas the control embryos presented a normal development. However, hatching was delayed at higher concentrations of the anaesthetic with 33.4% and 70.8% of the alive embryos unhatched at 0.64 and 0.77 mM ($p < 0.01$) respectively by 72 h (Fig. 3a).

The most frequent effects ($p < 0.05$) to LDC exposure were side-laying of larvae due to lethargy, tail malformation, scoliosis and severe bradycardia (Fig. 2f, 2g, 4a and 5a). At 96 h, 30-95% of the larvae were side-laying and not actively swimming (even when prodded) in the 0.25 to 0.64 mM treatments ($p < 0.05$) whereas all the zebrafish larvae were actively swimming in the 0.11 mM treatment. Significantly marked decreases ($p < 0.01$) in the zebrafish embryonic heart rate were also noted in 48 hpf to 120 hpf embryos exposed to 0.38 mM (88 beats/min), 0.51 mM (72 beats/min), or 0.64 mM (58 beats/min) of LDC indicating bradycardia (Fig. 5a). A significant occurrence of neurotoxicity ($p < 0.05$) as evidenced by scoliosis or curved body and tail deformation was observed after 96 h in 22.2% of the alive embryos exposed to 0.64 mM (Fig. 4a). Pericardial edema was also significantly induced in the 0.64 mM ($p < 0.05$) and 0.77 mM treatments in 22.2% (Fig. 4a).

Cyclophosphamide

The lethal concentrations of CYC (2.1-4.59 mM) adversely affected hatchability in exposed zebrafish embryos ($p < 0.001$). The overall hatchability of the embryos declined as the exposure concentration increased. At 48 h of exposure, more than 90% of embryos had hatched in the control, and 0.76-1.34 mM treatments; 50% in 2.1 mM; while only 20 and 15% of embryos hatched in the 3.44-4.59 mM treatments respectively (Fig. 3b). At 72 h, when non-hatchability was < 10% in control, and 0.76-1.34 mM treatments, 42.2% of alive zebrafish embryos were unhatched at 2.1 mM; 64.6% unhatched at 3.44 mM and none hatched at 4.59 mM. At 96 h, 45% of the larvae were still unable to hatch in the 2.1 mM treatment. The non-hatched embryos from 3.44-4.59 mM treatment presented a rigid chorion at 72 h but died without being able to hatch after 96 h of exposure.

The most common abnormalities ($p < 0.05$) caused by CYC exposure were observed at the lethal concentrations and include edema of the pericardium, malformed tail and curved spine. Bradycardia was also induced in the embryos following CYC exposure (Fig. 5b). At 48 h, when there was no mortality in the treatment groups, 30 and 60%

of embryos had pericardial edema in the 3.44, and 4.59 mM treatments respectively (data not shown). Neurotoxic effects, tail malformation and bent spine, were also significantly induced ($p < 0.05$) at 3.44 and 4.59 mM respectively (Fig. 2h) at 48 h in 20% of the embryos (data not shown). At 72 h, 15.5% of the alive embryos had pericardial edema at 2.1 mM; 43.7% had edema in 3.44 mM and all the non-hatched alive embryos at 4.59 mM (data not shown). After 96 h, pericardial edema and yolk edema were found respectively in 26.7% ($p < 0.05$) and 18.3% ($p < 0.001$) of the alive embryos exposed to 2.1 mM (Fig. 4b). Sublethal levels of the anticancer drug (0.76-1.34 mM) did not induce any significant abnormalities in the embryos and the adverse effects ($p < 0.05$) were only seen in form of lethargy in 26.1% of the larvae exposed to 1.34 mM after 96 h (Fig. 4b). Side-laying of the fish increased with concentration with 45% of alive larvae exposed to 2.1 mM inactive after the exposure period.

At 48 and 72 h when mortality was < 10% in the 2.1 mM treatment, about 10-15.5% of the embryos had their heads stuck in a rigid and opaque chorion (Fig. 2j) and were unable to free themselves with 18.3% of the alive larvae significantly affected ($p < 0.05$) by 96 h. (Fig. 4b). This partial dechorionation was only noted in the CYC exposure and was also seen in the embryos exposed to the higher concentrations, although occurrence was insignificant. Significant differences ($p < 0.05$) between the treatments and the control group, in terms of the heart rate, were only noted (from 48 to 96 h) when the embryos were exposed to the lethal concentrations (Fig. 5b).

Ifosfamide

Exposure of the embryos to IFS had no effects on hatching with more than 80% of *D. rerio* embryos hatched at 48 h or 72 hpf, and hatchability was 100% by 96 h at all tested concentrations (Fig. 3c). IFS had little or negligible effects on the development of the 24 hpf embryos at the tested concentrations; less than 10% of the embryos had pericardial edema. However, noticeable behavioural changes ($p < 0.05$) with regards to the side-laying of the larvae were observed in the treatments, 0.76-2.3 mM after 96 h (Fig. 4c). The heart rates of the larvae were not significantly affected ($p > 0.05$) by IFS exposure with the average heartbeats ranging between 150 and 170 beats/min (Fig. 5c).

Sulfamethoxazole

In sulfamethoxazole exposure, hatching was found to be enhanced at 48 h ($p < 0.05$), increasing with the concentration of the antibiotic except for the 0.79 mM treatment (Fig. 3d). While 25% and 35% of embryos were hatched in the control and lowest tested concentration (0.09 mM) respectively by 48 h, 85% of embryos were observed to have hatched in the highest treatment group (1.58 mM). However, this latter treatment induced the highest occurrence of abnormalities ($p < 0.01$): pericardial edema (30%), yolk sac edema (30%), curved body (30%), and side-laying (60%) in the exposed larvae after 96 h (Fig. 4d). A significant decrease ($p < 0.05$) in heart rate of zebrafish was also noticed at the highest concentration after 96 h (Fig. 5d). The lower concentrations of the sulfonamide had little or no effects ($p > 0.05$) on the development of the embryos (< 10% malformed embryos) (Fig. 4d), and significant effects ($p < 0.05$) on embryonic heart rates were only observed at 24 h of exposure (Fig. 5d).

Erythromycin

Although, hatching of zebrafish embryos was enhanced ($p < 0.01$) at the higher concentrations of erythromycin (0.068-0.136 mM) at 48 h, over 90% of the embryos exposed to ERY at all tested concentrations and in the control, had hatched by 72 h (Fig. 3e). Moreover, the macrolide did not cause any malformation nor induced any behavioural response in the embryos throughout the duration of exposure. ERY, however, significantly decrease ($p < 0.05$) the heart rate of the embryos in all treatments at 24 h and in the highest treatment at 48 h while no significant effects on the heart rate were seen in the 96 and 120 hpf larvae (Fig. 5e).

Amoxicillin

AMX had no effects on the hatching of zebrafish embryos with 100% hatchability observed in all the treatments and control by 48 h (Fig. 3f). Likewise, no abnormalities and behavioural effects were induced by AMX. Significant differences ($p < 0.05$) in the heart rate of embryos between the control and treatments (1.09-2.73 mM) were only noticed at 48 h of exposure (Fig. 5f).

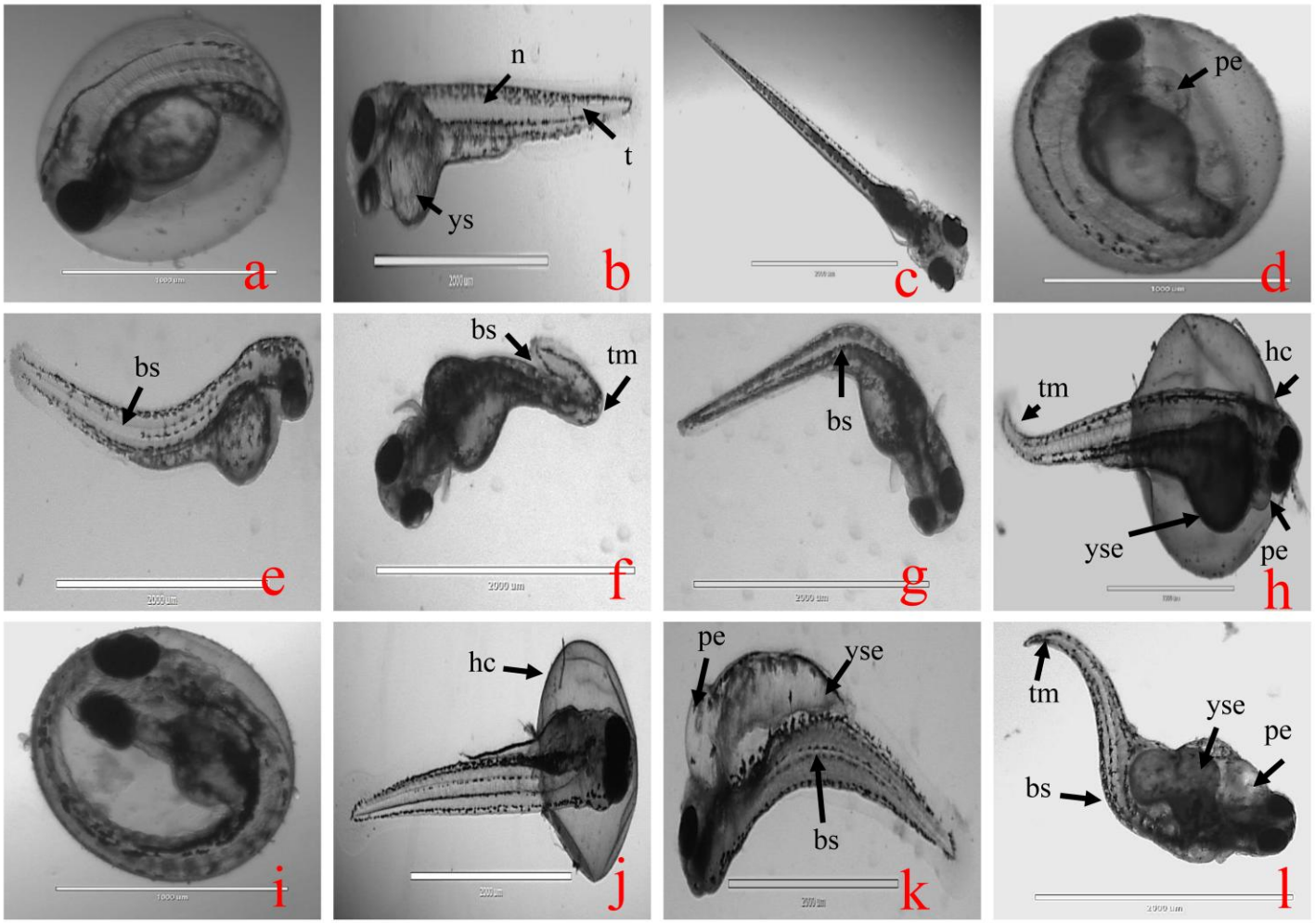

Figure 2. Phenotypic changes in zebrafish embryos following pharmaceutical exposure: (a) unhatched 48 hpf control embryo; (b) hatched 72 hpf control larva with normal notochord or spine; (c) 96 hpf control; (d) unhatched 48 hpf embryo at 3.44 mM cyclophosphamide; (e) 48 hpf larva at 0.51 mM lidocaine; (f & g) 72 hpf larvae at 0.64 mM lidocaine; (h) 72 hpf larvae at 3.44 mM cyclophosphamide; (i) unhatched 96 hpf and (j) hatched 96 hpf larva at 2.1 mM cyclophosphamide; (k) 96 hpf larva at 1.58 mM sulfamethoxazole; (l) 96 hpf larva at 0.77 mM lidocaine. Abbreviations: n, notochord; p, pericardium; t, tail; ys, yolk sac; bs, bent spine (scoliosis); tm, tail malformation; pe, pericardial edema; yse, yolk sac edema; hc, head stuck in chorion.

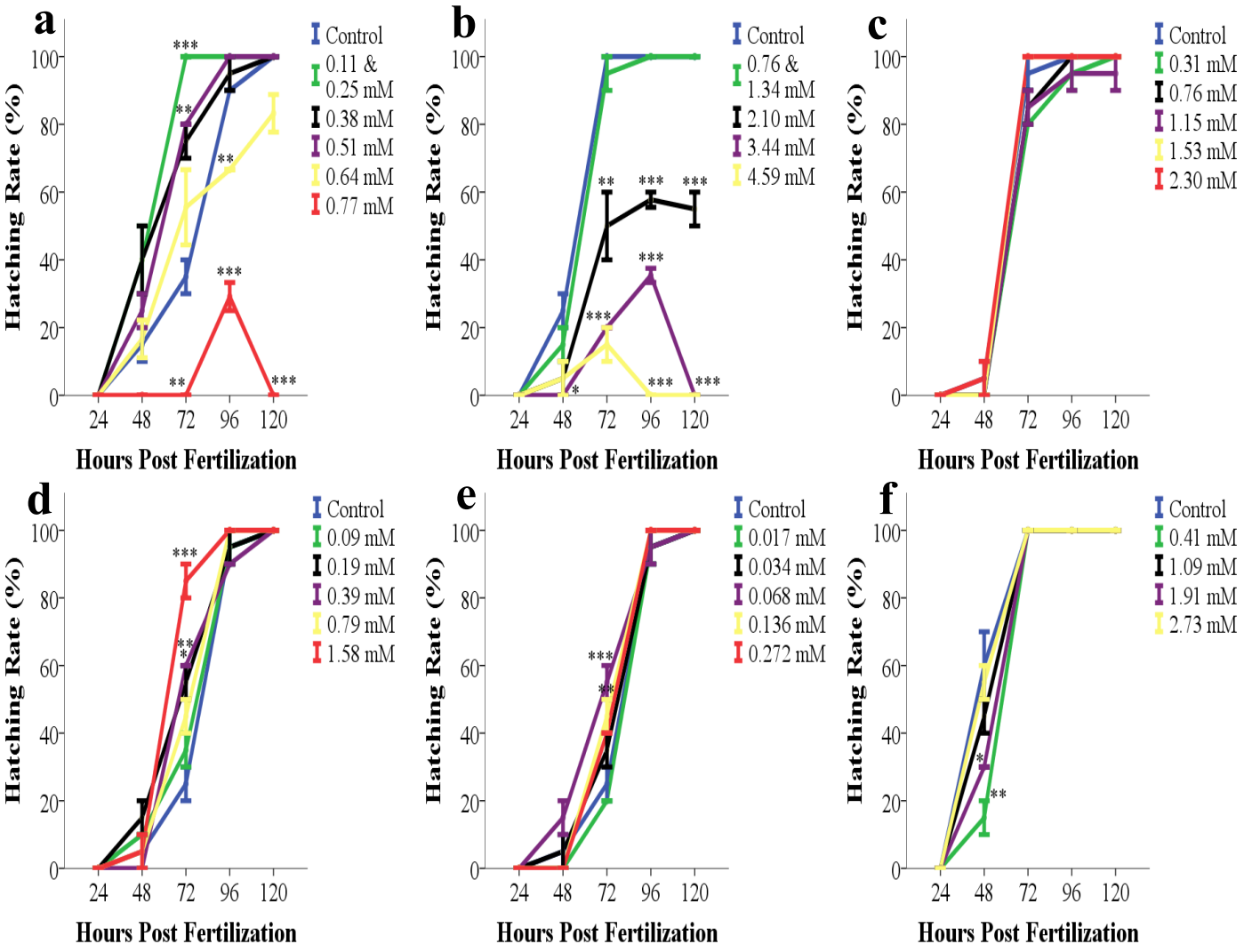

Figure 3. Hatching rate in 24 hpf embryos exposed to (a) lidocaine; (b) cyclophosphamide; (c) ifosfamide; (d) sulfamethoxazole; (e) erythromycin; and (f) amoxicillin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bar represents SE.

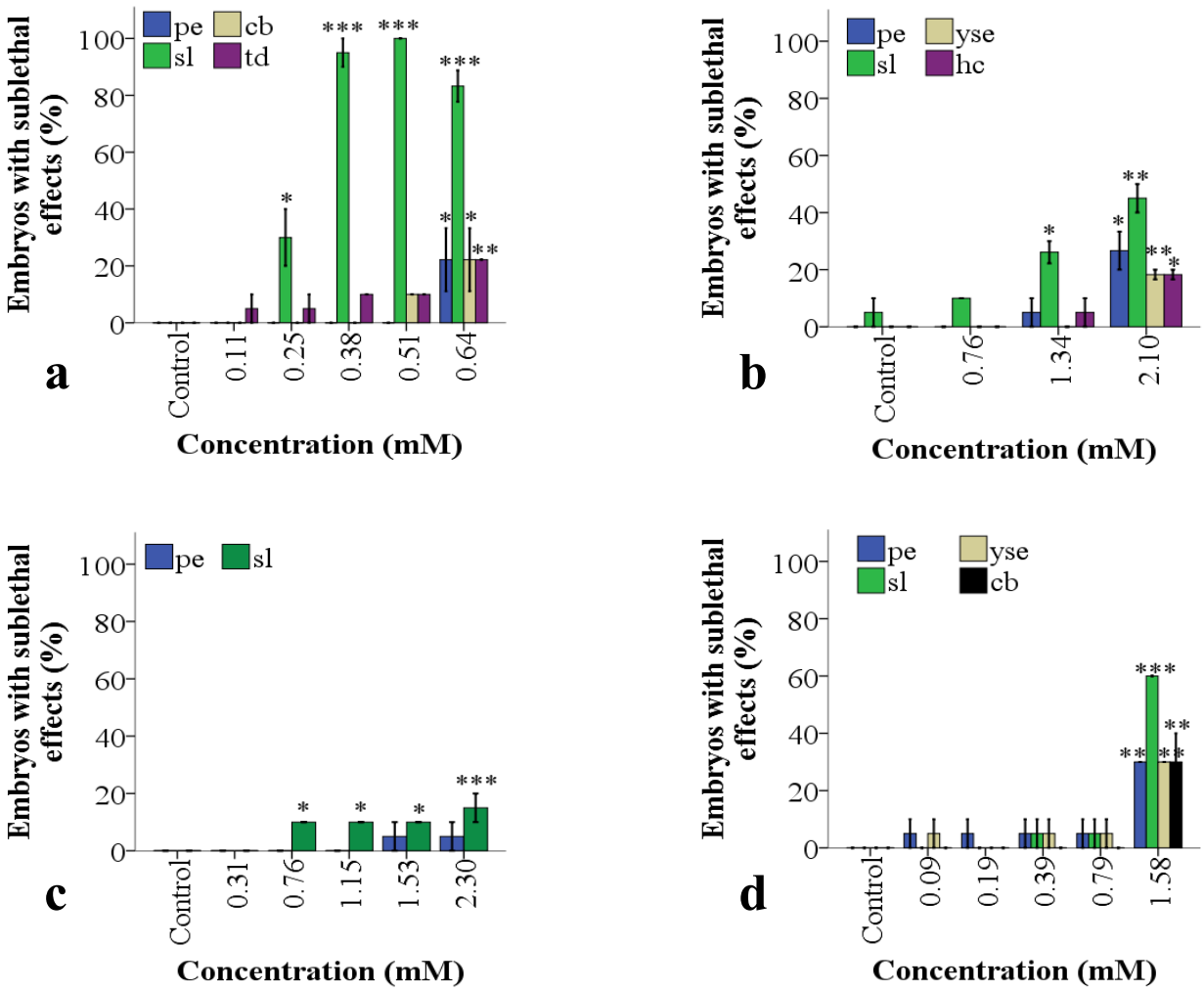

Figure 4. Frequency (%) of zebrafish embryos affected by sublethal endpoints after 96 h exposure to lidocaine (a); cyclophosphamide (b); ifosfamide (c); and sulfamethoxazole (d). (pe) pericardial edema; (sl) side-laying; (cb) curved body; (hc) head stuck in chorion; and (td) tail deformation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bar represents SE.

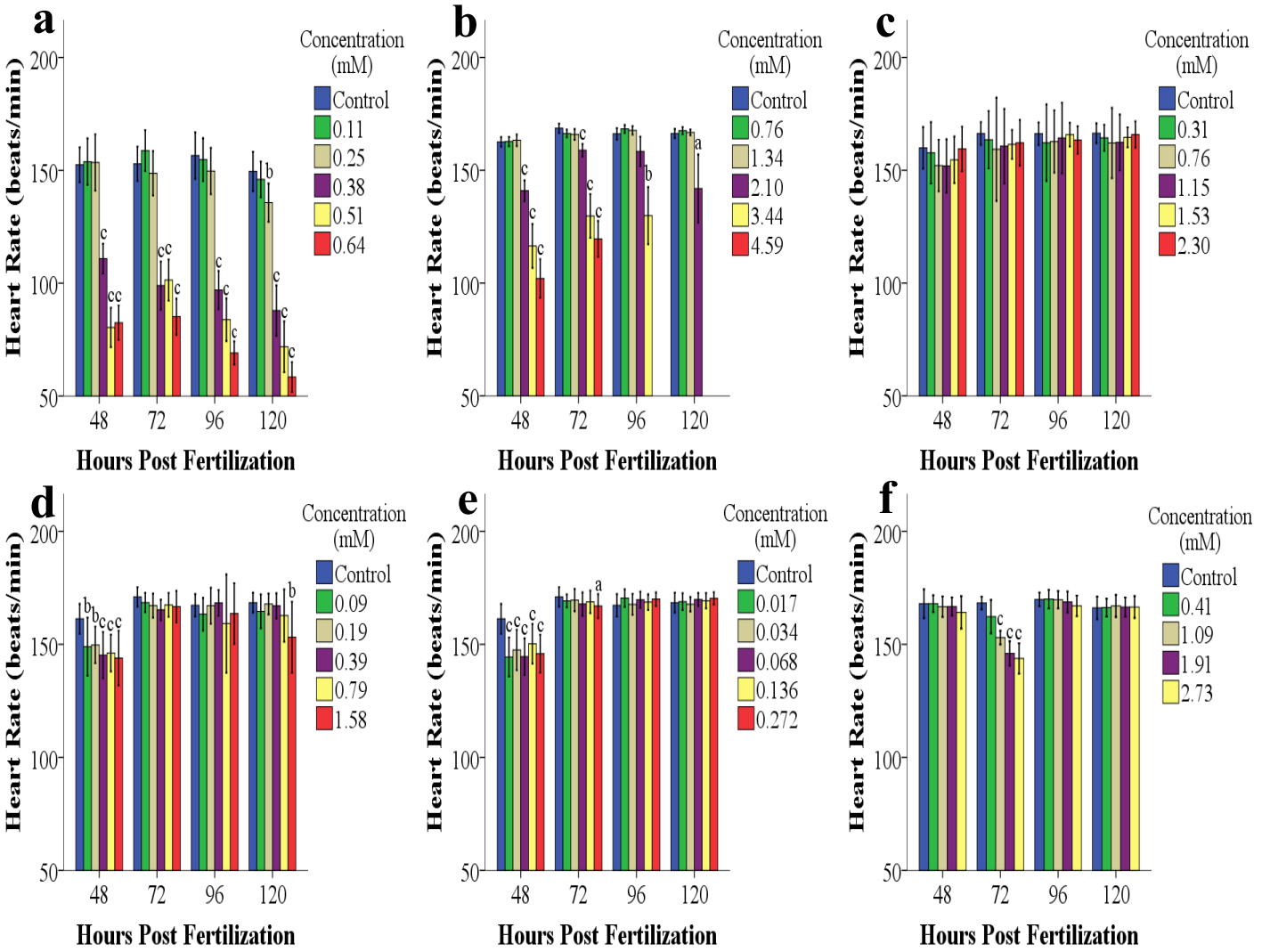

Figure 5. Effects of lidocaine (a); cyclophosphamide (b); ifosfamide (c); sulfamethoxazole (d); erythromycin (e); and amoxicillin (f) on heart rate in zebrafish embryos ($^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$). Error bar represents SD

CAT enzyme activity

In the zebrafish embryos exposed to LDC, CAT activity was significantly inhibited at 96 h in the 0.64 mM treatment but a significant increase in CAT activity was noticeable at the sublethal concentrations, 0.25-0.51 mM (Fig. 6a). Likewise, CAT activity was significantly increased in the 120 hpf larvae in IFS exposure but at the highest sublethal concentration (Fig. 6c). However, no significant changes in CAT activity were observed at 96 h in the larvae exposed to the sublethal levels of CYC and ERY (Fig. 6b and 6d). Unfortunately, due to loss of materials no results could be presented for CAT activity in the larvae exposed to SUF and AMX.

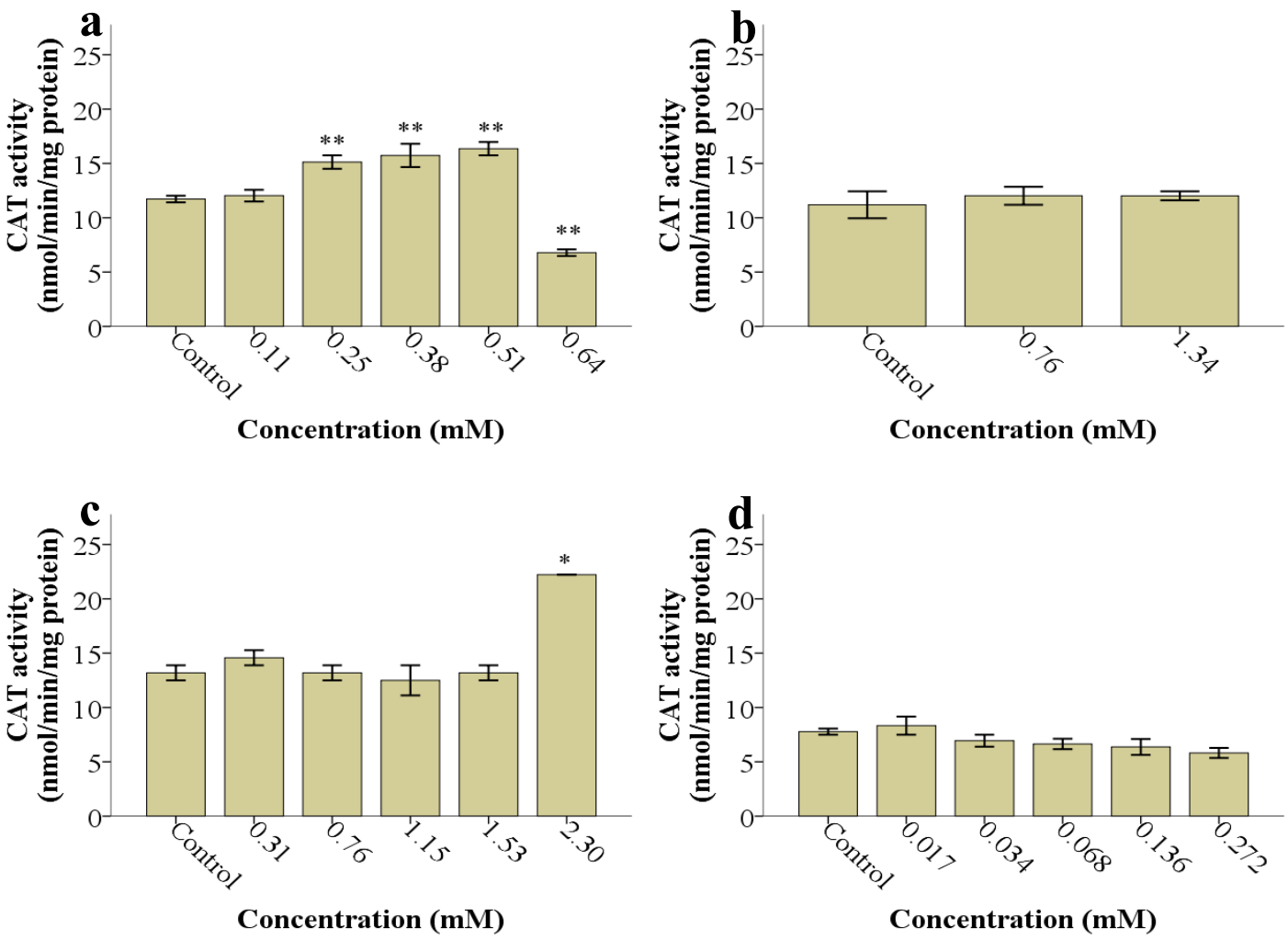

Figure 6. CAT activity in zebrafish embryos after 96 h exposure to lidocaine (a); cyclophosphamide (b); ifosfamide (c); and erythromycin (d). *$p < 0.05$, **$p < 0.01$. Error bar represents SE

mRNA expression levels of antioxidant enzymes

Based on qPCR measurements, the oxidative stress related genes were significantly regulated in the zebrafish embryos after exposure to the test pharmaceuticals. Table 3 shows the fold change or relative mRNA expression levels of the antioxidant enzymes. CAT, SOD1 and SOD2 genes were significantly upregulated ($p < 0.05$) in the embryos exposed to 0.25-0.51 mM LDC, and significantly downregulated ($p < 0.01$) at the 0.64 mM of LDC. Likewise, an upregulation of CAT and SOD1 genes was induced in the embryos as a result of CYC exposure while the expression of SOD2 gene was significantly increased only at 1.34 mM of CYC. A significant upregulation of the CAT gene was also noticed in embryos exposed to 2.3 mM and 1.58 mM of IFS and SUF respectively. However, the expression of SOD1 was unaffected in the embryos exposed to both pharmaceuticals. SOD2 gene expression significantly increased at 2.3 mM of IFS compared to the control group, and a significant decrease in the expressions of the gene was observed at 0.31 mM and 0.09 mM of IFS and SUF respectively.

Significant increases ($p < 0.05$) in CAT and SOD1 gene expressions were induced in the larvae at 0.272 mM of ERY while SOD2 gene was significantly downregulated in the zebrafish following the antibiotic exposure. The antioxidant enzymes gene expression levels of CAT, SOD1 and SOD2 were all significantly downregulated ($p < 0.01$) in the larvae exposed to AMX.

Table 3. The average fold change in mRNA expression levels of the antioxidant enzymes using qRT-PCR

Concentration (mM)	CAT	SOD1	SOD2
Lidocaine			
Control	1.00 ± 0.01[a]	1.03 ± 0.03	0.95 ± 0.02
0.25	2.17 ± 0.10***	1.05 ± 0.01	1.19 ± 0.08
0.38	2.63 ± 0.07***	2.28 ± 0.03***	2.41 ± 0.25*
0.51	2.79 ± 0.09***	2.12 ± 0.02***	3.75 ± 0.08**
0.64	0.49 ± 0.03**	0.48 ± 0.03**	0.41 ± 0.02***
Cyclophosphamide			
Control	0.99 ± 0.06	1.08 ± 0.04	0.98 ± 0.01
0.76	1.66 ± 0.01*	1.38 ± 0.04	1.17 ± 0.05
1.34	3.75 ± 0.09***	3.95 ± 0.10***	3.29 ± 0.10***
2.10	3.36 ± 0.12**	7.22 ± 0.28***	1.14 ± 0.04
Ifosfamide			
Control	4.12 ± 2.18	1.12 ± 0.08	1.12 ± 0.06
0.31	8.20 ± 0.34	0.72 ± 0.02	0.43 ± 0.01***
2.30	18.98 ± 2.00**	1.49 ± 0.14	2.59 ± 0.06***
Sulfamethoxazole			
Control	1.33 ± 0.27	1.01 ± 0.04	1.07 ± 0.04
0.09	1.38 ± 0.09	1.02 ± 0.08	0.77 ± 0.01**
1.58	2.27 ± 0.07*	1.08 ± 0.12	0.97 ± 0.02
Erythromycin			
Control	1.33 ± 0.27	1.01 ± 0.04	1.07 ± 0.04
0.017	1.89 ± 0.07	1.00 ± 0.04	0.64 ± 0.03***
0.272	2.65 ± 0.03*	1.27 ± 0.07*	0.74 ± 0.01**
Amoxicillin			
Control	1.01 ± 0.02	1.03 ± 0.03	1.06 ± 0.04
0.41	0.17 ± 0.01***	0.02 ± 0.00**	0.12 ± 0.00***
2.73	0.40 ± 0.01***	0.40 ± 0.01**	0.49 ± 0.02***

[a]Fold change ± SE, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

DISCUSSION

Little is known regarding the risk posed by LDC toward aquatic species. According to a safety data report (AstraZeneca, 2016) a 96 h LC_{50} of 0.45 mM has been reported for the anaesthetic in adult zebrafish which is very close to the value obtained for the 24 h embryos in this study. Interestingly, the significant occurrence of neurotoxicity and cardiovascular effects in the fish embryos at lethal and sublethal levels of LDC correlates with its adverse effects in mammals (Drasner *et al*., 1994; Kovacic and Somanathan, 2011). The enhancement of embryo hatching, and its delay caused by LDC exposure may be attributed to different toxic mechanisms. Yamagami (1981) suggested that such alterations in hatching could arise due to the induction of abnormal functioning of the enzyme chorionase and the inability of the emerging larva to break the eggshell.

The median lethal concentration of 8.4 mM reported for CYC in 2.5 hpf embryos exposed for 72 h (Weigt *et al*., 2011) is much higher than the 72 h LC_{50} (3.75 mM) obtained for CYC in this study. The difference may be attributed to the presence of chorion at early stages which shield the early embryos from teratogen actions (Ali *et al*., 2011). The abnormalities induced in 20-60% of 48-72 hpf embryos at the highest CYC treatment, in agreement with Weigt *et al*. (2011), demonstrate the ability of zebrafish embryos to activate proteratogenic compounds without any addition of an exogenous metabolic activation system. Zhu *et al*. (2014) reported a significant occurrence of cardiotoxic manifestations in 48 hpf zebrafish embryos exposed to CYC for 24 h which is in consonance with findings obtained at 72 hpf in this study. Pericardial edema or effusion induced by CYC has also been reported in humans (Katayama *et al*., 2009; Nakamura *et al*., 2010).

The head stuck in chorion phenomenon noticed in a significant number of embryos at the highest CYC levels was earlier reported in pikeperch eggs exposed to tannic acid and was attributed to possible inhibition of chorionase activity by the test compound (Demska-Zakes *et al*., 2005). In the aquatic environment, few studies have reported the effects of antimicrobials including SUF, AMX and ERY in *D. rerio* embryos probably as a result of the low toxicity shown generally by antibiotics to fish (Oliveira *et al*., 2013). The enhancement of hatching by SUF and ERY in this study may be due to the induction of abnormal functioning of chorionase activity (Yamagami, 1981). However, a delay in hatching was reported in 5 hpf embryos exposed to 0.1-100 µg/L SUF (Liu *et al*., 2020). The increase in the defects especially pericardia edema observed in 30% of embryos exposed to SUF from 72-96 h may be due to the absence of the chorion to offer the embryos protection to some extent by acting as a barrier to certain chemicals (Braunbeck *et al*., 2005). Lin *et al*. (2013) reported a similar occurrence in juvenile zebrafish (10-13.33%) after 1-2 hpf embryos were exposed to 3.95 µM of SUF for 96 h.

However, the higher toxicity shown by SUF toward the early life stages of the fish in the earlier study, suggests that the chorion is unlikely to have much effect on their diffusion.

Zebrafish has been identified as an alternative model for investigating congenital hypothyroidism (Porazzi *et al*., 2009). The curvature of the body defect in the zebrafish exposed to SUF in this study suggests that the drug may affect the development of the nervous system. This is in consonance with an earlier study that reported tail bending deformity in zebrafish embryos exposed to sulfonamides attributing it to hypothyroidism (Lin *et al*., 2013). Thyroxine is involved in regulating neural signalling in zebrafish early embryonic stages, promoting the development of the nervous system (Yonkers and Ribera, 2008). Bradycardia and pericardial effusion have been listed as some of the cardiovascular signs or symptoms of hypothyroidism in humans (Khaleeli *et al*., 1982; Levey and Klein, 1994). The cardiovascular effects of SUF in this study, although not fully understood, may also be linked to hypothyroidism and further studies need be carried out to ascertain this.

Oxidative stress is a deleterious process caused by an imbalance between oxidants and antioxidants in steady state resulting in damage to cellular components including proteins, lipids and DNA (Doi *et al*., 2011). Sources of oxidants or reactive oxygen species, O_2^- and H_2O_2, are functionally and spatially related to the production and cellular localization of natural antioxidant enzymes such as SOD and CAT (Gobe and Crane, 2010). SODs detoxify O_2^- into H_2O_2. H_2O_2 is then converted to water and molecular O_2 by CAT. The balance between ROS production and antioxidant system leads to regulated intracellular steady state levels of ROS in aerobic organisms (Sies, 1997).

A significant upregulation of the gene expressions of SOD1, SOD2 and CAT and the consistency between the mRNA expression levels of CAT and its enzyme activity, at sublethal levels of LDC (0.25-0.51 mM), suggest that these antioxidants may play an important role in preventing LDC-induced neurotoxicity as side-laying and bradycardia were the only significant phenotypic effects elicited in the larvae. The absence of malformed heart and the neurotoxicity suppression (occurring in ≤ 10% of larvae) against possible ROS attack is possibly via O_2^- dismutation and H_2O_2 scavenging.

Furthermore, this study demonstrated a significant decrease in the amounts of transcripts of antioxidant enzymes SOD1, SOD2, and CAT as well as a corresponding decrease in CAT activity in embryos exposed to the higher LDC concentration (0.64 mM) at 96 h post treatment. This indicates that, the generation rate of ROS was much higher than the clearing capacity of the antioxidant systems, causing oxidative stress and possibly was responsible for the mortality, cardiovascular toxicity and neurotoxicity observed in the fish after 96 h of exposure.

Recent studies have shown that mitochondria are the main source of ROS and the major target of ROS-induced damage (Ravikumar *et al*., 2010). Mitochondria have been known to be the most important cellular source of O_2^- and H_2O_2 in aerobic organisms (Okamoto *et al*., 2016). The toxicity of LDC in humans has been associated with ROS induction (Kovacic and Somanathan, 2011). LDC penetration of the mitochondria has been identified with the outcome of significant inhibition of mitochondrial function, alteration of gene expression, induction of ROS and eventual apoptosis in cells (Kovacic and Somanathan, 2011; Lee *et al*., 2016). A strong evidence for neurotoxicity of local anaesthetics, such as LDC and bupivacaine, through apoptosis triggered by ROS production has been reported (Park *et al*., 2005). Since dysfunctional mitochondria are central to the formation of excess ROS and are known key intracellular targets for LDC (Okamoto *et al*., 2016), it is possible that the downregulation of SOD2, a mitochondrial SOD, at 0.64 mM LDC in this study, had a major part to play in the cardiotoxic and neurotoxic abnormalities seen in the zebrafish larvae via disruption of the mitochondrial antioxidant system.

The cellular mechanism of toxicity or cardiotoxicity of anticancer agents, CYC and IFS, in animal models is linked with the production of high levels of ROS by their metabolites, culminating in oxidative stress (Nepomuceno, 2011; Lamberti *et al*., 2014). The substantial upregulation of CAT and most especially SOD1 gene expressions at 2.1 mM of CYC indicates very high levels of ROS which the antioxidant systems are unlikely unable to cope with, possibly resulting in the CYC-induced mortality and teratogenic effects (hatching failure, yolk sac edema and cardiotoxic manifestations) seen in the larva at 96 h post treatment.

CYC, at sublethal and non-toxic (at organism level) concentrations (0.76-1.34 mM) upregulated the mRNA expression levels of CAT, SOD1 and SOD2, an indication that these antioxidants may play a vital role in protecting the exposed embryos from the teratogenic effects of the anticancer drug. The induction of only CAT gene expression at the lowest CYC concentration suggests that CAT could be involved in a more critical role in the detoxification process than SOD1 and SOD2. The teratogenic risks posed by IFS exposure is likely to have been suppressed by the scavenging and dismutative actions of CAT and SOD2 respectively as shown by the significant upregulation of their gene expressions with the expression of CAT in concordance with an increase in its enzyme activity in the exposed larvae at the highest tested concentration of IFS.

An upregulation of the transcriptional expression levels of SOD1 and CAT (at the highest ERY treatment) shows that the antioxidants may be involved in the prevention of ERY induced-embryotoxicity. In addition, the significant induction of CAT mRNA expression in the fish at the highest concentration of SUF cannot rule out the possibility

that the cardiotoxic and neurotoxic effects elicited in the fish could be due to ROS accumulation as the antioxidant response may not be enough to counteract the accumulated ROS. The significant downregulation of the antioxidant genes especially SOD2 at the non-toxic concentrations of IFS, SUF, ERY and AMX, although not clearly understood, correlates with the absence of embryotoxicity in the zebrafish.

The induction of the mRNA expression levels of the antioxidants and CAT activity by the sublethal concentrations of LDC, CYC, IFS, and ERY in this study suggest that an oxidative stress response (at a higher level of biological organization) is possible in the zebrafish exposed for a longer period or to a higher concentration of the pharmaceuticals (Gunnarsson *et al*., 2009). However, the evaluation of a battery of biomarkers would be necessary to assess the likelihood of these occurring.

CONCLUSIONS

The multi-level effect approach adopted in this study provides a better understanding of the mechanisms of toxicity employed by the pharmaceuticals especially in LDC, CYC and SUF exposures and the evaluation of more nonspecific or specific biomarkers would have provided more details on the pathways. The sublethal concentrations of LDC, CYC, IFS and ERY activated protective antioxidant defenses in the embryos in this study. Although, these concentrations are above the levels measured in the aquatic environment, their significant upregulation of the antioxidants at the transcriptomic and cellular levels in this study suggest possible phenotypic or oxidative stress response in the fish after a chronic exposure to the pharmaceuticals. This study shows the essence of evaluating stress biomarkers and emphasizes the need for chronic studies in order to have a more meaningful assessment of pharmaceutical effects on aquatic organisms.

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